

University Proposal # /

FOR PASSHE OFFICE USE ONLY: FPDC proposal #

Project Title: Exploration of transmission and gene expression among the phylogenetic lineages of invasive *Escherichia coli* in the housefly (*Musca domestica*) vector

RFP Category: 1-A

Total Grant Amount Requested from FPDC: \$ 7740

Discipline: Biology

Sub-Discipline: Microbial Genetics

Project Director (name, position, department, university, telephone number, and e-mail address):

Alyssa C. Bumbaugh, Assistant Professor of Biology, Department of Biology, Shippensburg University  
Phone: 717 477 1593; e-mail: acbumbaugh@ship.edu

Faculty Status (see definitions below):

Tenured

Probationary

Non-Tenure Track

Other Participants (names, departments, e-mail addresses):

N/A

IRB Status:

Approved (IRB #

)

Pending

N/A

ABSTRACT (one paragraph of approximately 150 words in non-technical language):

Successful bacterial pathogens must colonize and survive within a host as well as transmit to a new susceptible host. By using co-culture methods, we will investigate the nature of the relationship between the invasive *E. coli* bacteria and the housefly vector (*M. domestica*). The invasive *E. coli* harbor numerous genes that are involved in the establishment of human disease and subsequent pathogenesis. This project will reveal if there is a genetic basis for the prevalence of certain serotypes isolated from disease cases through determination of carriage rates on the housefly vector and development of a gene expression library. Experiments will also discover if an infection process occurs within the vector. Collectively, the results could elucidate differences in bacterial gene composition or expression patterns which could provide targets for diarrheal disease prevention through vector control or vaccination strategies.

Endorsement:



Chair, University Faculty Professional Development Committee

15 FEB. 2010

Date

Endorsement:



University President

15 Feb 2010

Date

**Faculty Status Definitions:**

Probationary Non-Tenured Faculty - a faculty member who is appointed to a tenure track position and who has not been granted tenure.

Non-Tenure Track Faculty - a faculty member who is appointed to service in a position in which service will not be credited toward tenure, for example Temporary Part-Time or Temporary Full-Time or Regular Part-Time faculty.

## Background and Significance

From an evolutionary perspective, if a bacterial pathogen is to be successful, it must employ tactics that allow for colonization, survival in a host and the external environment, and dissemination to a susceptible host. In the broad knowledge base of microbiology, there are many reports of novel and evolving strategies by which these disease-causing microbes have perfected these tactics. One survival tactic employed by several unrelated bacteria is the ability to invade or take up residence in host cells. This permits a safe haven for the pathogens as they are hidden from the host immune system. Subsequently, the pathogen has access to a potentially nutrient rich environment which can lead to increases in bacterial numbers and establishment of an infection within the host.

This proposal focuses on the invasive *Escherichia coli* which includes *Shigella* species and certain *E. coli* serotypes designated as the Enteroinvasive *E. coli* (EIEC) pathovar. Collectively, these pathogens are responsible for the death of more than 1.1 million people each year worldwide (9) and cause more than 400,000 cases of illness per year in the United States (11). Invasive *E. coli* infections are transmitted by the fecal-oral route usually as a result of direct person-to-person transfer or through contact with or ingestion of contaminated food and water (2). The infectious dose is very low with ingestion of as few as 10 bacteria causing symptomatic infections (2).

*Escherichia* and *Shigella* are closely related members of the Enterobacteriaceae; however, they each cause a clinically distinct disease and differ in their clinical biochemical profiles. *Shigella* species are invasive and cause bacillary dysentery, whereas within the *Escherichia*, only the EIEC have the same ability. The genetic basis for the invasive ability is conferred by the gain of a large plasmid (a mobile DNA molecule) carrying genes encoding virulence factors responsible for the disease symptoms and pathogenesis. The acquisition of this virulence plasmid appears to have occurred early on in the evolutionary history of the *Escherichia* and *Shigella*. Regulation of the plasmid-borne genes is temperature dependent (4) with some products being produced at ambient temperatures and others produced when the bacteria are exposed to human body temperature. Additionally, the spread of mobile blocks of virulence genes known as pathogenicity islands, and loss-of-function mutations caused by large genomic deletions (called "black holes") have enhanced virulence characteristics in *Shigella* and EIEC.

There are four species of *Shigella*: *S. boydii*, *S. dysenteriae*, *S. flexneri*, and *S. sonnei*, that have been recognized historically because of the severity of disease and their clinical importance. The four species are identified and distinguished by biochemical traits and the expression of specific cell surface antigens. The prevalence of the various strains in disease varies geographically and has changed historically. At the present time, *S. flexneri* 2A is the most prevalent strain in developing countries, whereas *S. sonnei* infections continue to account for most shigellosis in industrialized nations. While not as well represented in the scientific literature as the Shigellae, the EIEC have been involved in several large outbreaks of acute gastroenteritis in the United States (6, 7). In the developing world, EIEC infections contribute to endemic rates of diarrheal disease; enteroinvasive strains are typically isolated in 1 - 5 % of the cases of acute diarrhea in children (3, 5), although incidence rates vary with season and socio-economic conditions.

Even though biochemical and clinical differences exist between the *Escherichia* and *Shigella*, recent molecular evidence (12, 13) indicates that the classification of these bacteria is artificial which is why this proposal collectively refers to these pathogens as the invasive *E. coli*. Molecular analyses have also shown that there are multiple origins of the invasive lineages within the history of the *Escherichia* genus (14). My previous research efforts support these reports (1) and ongoing studies in my laboratory seek to examine the differences in virulence gene distribution both within and between the nine identified invasive groups.

Shigellae colonize only humans and non-human primates so there are no alternative species of animal reservoirs. It has been reported that houseflies (*Musca domestica*) serve as a mechanical vector

for the spread of *Shigella* (10). It is logical that the housefly serves as a mechanical vector for shigellosis since there is cohabitation with humans and a tendency to breed in human feces and refuse. Due to sanitation and fly control measures, the incidence of shigellosis has decreased in the United States. A recent finding (James Nataro, personal communication) indicates that the invasive *E. coli* are the leading bacterial cause of diarrheal disease in children at Gates Foundation study sites in developing countries. Nataro (personal communication) also noted the prevalence of flies in and around the latrines, markets, and homes at the study sites. When these flies are trapped and cultured, *Shigella* can always be detected.

Investigating the nature of the relationship between invasive *E. coli* and *M. domestica* can allow us to determine if there is a genetic basis for the prevalence of certain serotypes isolated from disease cases. The bacteria are exposed to two temperature habitats as differences exist between the body temperatures of the housefly and human host. There is a potential difference in gene expression that could exist when the bacteria are being transmitted by the housefly. Elucidating these differences could provide targets for disease prevention through vector control or vaccination strategies.

**Objectives of the Study:** The goals of this proposed study are: 1) to determine if there are differences in the amount of carriage of invasive *E. coli* on the housefly based on phylogenetic groupings; 2) to establish if the housefly is solely a mechanical vector or if an infection process occurs within the fly; and 3) to produce a gene expression library of a representative invasive *E. coli* strain when it is residing with the housefly vector. It is documented that the invasive *E. coli* have different patterns of virulence gene expression at different temperatures (ambient versus human body temperature), and by conducting this experiment, we could determine if there are “vector specific” genes and “human host specific” infection genes.

### **Description of the Project**

**Differential carriage of invasive *E. coli* on the *M. domestica* vector.** The first stage of the project will focus on the development of a safe and effective way of implementing a housefly/invasive *E. coli* model system. All fly manipulations conducted with pathogens will be performed in secondary containment vessels to avoid the escape of the flies. Housefly (*M. domestica*) cultures will be commercially obtained and reared in the laboratory setting according to the supplier’s protocol. A small number of flies will be exposed to vials seeded with a non-pathogenic *Escherichia* isolate (laboratory strain K-12) during the initial development followed by representative isolates from each of the pathogenic lineages (a total of 16 isolates will be tested). The flies will be sedated and processed according to published procedures (15). Briefly, the procedure will utilize a wash method for the enumeration of surface bacterial carriage and a crush procedure for the enumeration of internalized bacteria. A portion of each sample (wash versus crush) will be plated (in triplicate) on differential and selective agar media (Eosin Methylene Blue and/or Hektoon Enteric Agar) to confirm isolation and allow for the enumeration of *E. coli* and *Shigella*. Additionally, positive colonies will be used for molecular verification of invasive *E. coli* by the presence or absence of PCR amplicons for both a chromosomal housekeeping locus (*mdh*) and an invasion plasmid-encoded virulence locus (*senA*). The results from this stage will provide data that can be used to determine differences in carriage rates between the invasive *E. coli* groups and provide some suggestive information about the internalization/infection process that might occur in the fly.

**Investigation of *M. domestica* infections.** In order to substantiate the internalization/infection data from the first stage of the project, we plan to obtain larvae that have been feeding and developing in the bacteria-laden growth medium. It is possible that the fly serves solely as a mechanical vector and transmits the bacteria via carriage on the sticky pads of the legs; however, if the fly is infected, transmission could occur as the fly regurgitates or defecates on a food source or household surface.

There is evidence that other Enterobacteriaceae are carried internally in a beetle vector and eliminated as part of the frass; however, there are conflicting reports about the carriage of invasive *E. coli* in the house fly. For this portion of the project, we plan to employ the same wash and crush procedures with the larvae as described above followed by cultural characterization. This would be performed on a smaller scale with 8 isolates being tested. If our data suggests internalization, future research plans will utilize green fluorescent protein (GFP) labeling in conjunction with fluorescence microscopy for confirmation and localization; however, this would be beyond the scope of the current proposed work.

**Development of a gene expression library.** To begin the examination of gene expression, we will construct a complementary DNA (cDNA) library from *S. flexneri* serotype 2A when it is being carried on the *M. domestica* vector. We will co-culture the vector and pathogen to allow for sufficient adherence to occur. The flies will be euthanized and dissected to minimize the amount of fly tissue in subsequent procedures. The Flexneri 2A RNA will be isolated and reverse transcribed to produce cDNA which can be used for downstream processes such as sequencing and microarray hybridization. The Flexneri 2A isolate will be used because the entire genomic sequence has been obtained (8, 16) and it represents a distinct evolutionary lineage within *E. coli* that is most often isolated from cases of diarrheal disease in developing nations. This phylogenetic lineage (referred to in the literature as the Group 3 Shigellae) has acquired several virulence factors which are absent in other invasive lineages. Perhaps this genetic background correlates with survival, virulence, transmission, or other pathogenic characteristics that provide the Group 3 Shigellae with a selective advantage in those environments. We intend that this cDNA library will be used in collaboration with other *Shigella* researchers to investigate gene expression using microarray analysis.

### **Opportunities for Learning and Advancement**

My graduate work focused on the establishment of evolutionary relationships of the invasive *E. coli* in order to develop and test hypotheses about gene acquisition and loss. The non-human mode of transmission for these pathogens seemed to be of minimal interest within the field, but was always intriguing to me. A meeting and subsequent conversations with colleagues at the University of Maryland reminded me of the impact of *Shigella* in developing nations and the need for control and prevention. This led our discussions to transmission mechanisms and questions about the differential isolation of certain serotypes. This project would serve to address some of those questions by experimentally testing the transmission model with isolates of different genetic compositions.

In addition to advancing the body of scientific knowledge, this project would afford new opportunities for me to explore a eukaryotic vector/microbial parasite interaction and learn techniques for RNA isolation and cDNA synthesis. The field of molecular biology changes rapidly with new techniques and technologies advancing the capabilities of basic research. Many of these new techniques are introduced in course content and textbooks; however, it would be advantageous for me to be able to share a more detailed research example as a case-based learning tool for my Genetics students. Working with RNA and gene expression are universal techniques that could be applied broadly in my future research projects.

This proposed project would allow for subsequent collaboration with larger, primary research laboratories as there would be a need to examine gene expression patterns using DNA microarrays (with Drs. James Nataro and David Rasko at the University of Maryland) upon completion of the cDNA library and perhaps a collaborative project to undertake the GFP labeling (with Dr. Shelley Payne at the University of Texas) and fluorescence microscopy. These collaborative projects would permit opportunities for networking and learning new skills and techniques.

Students involved in this project will further develop problem-solving and critical thinking skills as these will be paramount in the proposed work. This project would lead to presentation quality findings for the regional Allegheny Branch of the American Society for Microbiology (ABASM) and the General

Meeting of the American Society for Microbiology (ASM), a national microbiology meeting, as well as the potential for a faculty-student peer reviewed publication for intended submission to Applied and Environmental Microbiology.

### **Timeline**

A seven month time frame from June 2010 to January 2011 would be appropriate for the completion of this project. It will initially take about 2-3 weeks for *M. domestica* culture; however, once the population is established, we can maintain the fly culture in the laboratory. Since we are proposing to use Flexneri 2A to generate our expression library, this part of the project can begin once we have successfully developed the laboratory co-culture procedures and will be on-going. It is anticipated that we can test 3 strains per month to determine carriage rates. The remaining time will be used for the smaller scale experiments to establish whether or not an infection process occurs within the fly. This would entail measuring background levels of bacterial carriage, performing larval dissections, and molecular and cultural bacterial verification. Data analysis will also be an on-going process throughout the duration of the project.

An undergraduate student, Sarah Brown, has expressed interest in this project. She has basic microbiology experience and will be shadowing my current undergraduate researchers during the spring 2010 semester to become more familiar with molecular biology protocols.

### **Feasibility and Projected Outcomes**

Others have successfully reported the isolation and characterization of bacterial isolates from flies (15). We anticipate modeling their protocols with little to no modification to ensure success. The invasive *E. coli* groups are already established and current research in my laboratory is adding to the knowledge of gene acquisition in the different phylogenetic groups. It is possible that this work could begin to elucidate vector and host specific genes in the invasive *E. coli* much like those that have been identified for the causative agent of plague, *Yersinia pestis* as it is carried by the flea vector.

The project would further my professional development as I would gain experience with molecular techniques involving RNA and provide exposure of my work to regional and national audiences through meeting presentations (ABASM and ASM). These meetings would also enhance my presentation skill as well as those of the student researcher and provide the opportunity to explore and cultivate collaborative research efforts with primary research institutions. Another important outcome from this project would be the generation of preliminary data which would be incorporated into an external grant proposal to be submitted in December 2010 to the Ecology of Infectious Disease (EID) program in the Directorate of Biological Sciences (BIO) at the National Science Foundation (NSF).

I would also encourage an undergraduate student continuing with this project to apply for the ASM Undergraduate Research Fellowship in February 2011. This is a competitive award that would provide a summer stipend for student research, a two-year membership to the ASM organization, and partial funding for the for the student to present their research at the ASM General Meeting. Additionally, student submission of the fellowship application would also provide recognition for both myself and Shippensburg University. I anticipate that a joint student-faculty peer reviewed publication would result from this work which in turn would provide the student with increased marketability for a graduate program or research employment.

## Budget Summary

Project Budget	Proposed Grant	University Contribution	Other Revenue Sources	Totals
Salaries/Stipends	\$ 1250			\$ 1250
Student Wages	\$ 1820			\$ 1820
Benefits	XXXXXX	\$ 225		\$ 225
Supplies	\$ 2500			\$ 2500
Equipment	\$ 2170			\$ 2170
<b>TOTALS</b>	<b>\$ 7740</b>	<b>\$ 225</b>		<b>\$ 7965</b>

## Budget Notes

Salaries/Stipends – the requested amount will be used for 2.5 weeks of summer salary for the Principle Investigator. During this time, I will be working with and training the student researcher to maintain and manipulate the fly cultures, and begin molecular techniques by running background and control reactions. I will continue to work with the student throughout the summer; however, additional salary is not being requested for this additional time due to the amount requested for other budget items.

Student Wages– the requested amount will be used to compensate the student research for 250 hours of work on the proposed project.

Supplies – the requested amount includes: \$220 for fly media and culture (bottles, plugs, larvae, growth media); \$1800 for molecular (DNA and RNA isolation, cDNA kit, PCR mix, primers) and culture reagents (bacterial media) to be used in verifying the presence of the invasive *E. coli*; and \$480 for general laboratory consumables (tips, gloves, petri dishes, microfuge tubes, sterile pestles).

Equipment – the requested amount includes funds for a shaking platform, used sonicating bath, rotating tumbler (these items are needed for successful processing of the samples), and designated micropipettes for RNA work.

**Previous FPDC grants** – N/A

**Letters of endorsement** – N/A

**Any other contract documents** – N/A

## Curriculum Vitae

Alyssa C. Bumbaugh

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### Education

Pennsylvania State University	B.S.	Biology – Genetics and Developmental Option	1997
Pennsylvania State University	M.S.	Genetics	2000
Michigan State University	Ph.D.	Genetics	2003

### Professional Employment

<u>Institution</u>	<u>Title</u>	<u>Year</u>
Shippensburg University	Assistant Professor of Biology	2008-present
Pennsylvania State University – Altoona College	Assistant Professor of Microbiology	2004-2008
Centers for Disease Control and Prevention	ORISE Postdoctoral Researcher	2003-2004

### Teaching Experience/Courses Taught

Elementary Microbiology, Elementary Microbiology Laboratory, Introductory Microbiology, Introductory Microbiology Laboratory, Ecology of Infectious Disease, Genetics, Basic Biology, Principles of Biology II

### Area of Research Specialization

Molecular evolution of infectious disease, host/parasite interactions, molecular evolutionary genetics, genomics, invasive pathogens, molecular subtyping, foodborne pathogens

### Peer-Reviewed Publications

- Liu Y., P. Fratamico, C. DebRoy, **A. C. Bumbaugh**, and J. W. Allen. 2008. DNA sequencing and identification of serogroup-specific genes in the *Escherichia coli* O118 O antigen gene cluster and demonstration of antigenic diversity but only minor variation in DNA sequence of the O antigen clusters of *E. coli* O118 and O151. *Foodborne Pathogens and Disease* 5:449-457.
- Hyma, K. E., D. W. Lacher, A. M. Nelson, **A. C. Bumbaugh**, J. M. Janda, N. A. Strockbine, V. B. Young, and T. S. Whittam. 2005. Evolutionary genetics of a new pathogenic *Escherichia* species: *Escherichia albertii* and related *Shigella boydii* strains. *Journal of Bacteriology* 187:619-628.
- Whittam, T. S. and **A. C. Bumbaugh**. 2002. Inferences from whole-genome comparisons of bacterial pathogens. *Current Opinion in Genetics & Development* 12:719-725.
- Bumbaugh, A. C.**, E. A. McGraw, K. Page, R. K. Selander, and T. S. Whittam. 2002. Sequence polymorphism of *mip* and *dotA* alleles mediating invasion and intracellular replication of *Legionella pneumophila*. *Current Microbiology* 44:314-22.
- Reid, S. D., C. Herbelin, **A. C. Bumbaugh**, R. K. Selander, and T. S. Whittam. 2000. Parallel evolution of virulence in pathogenic *Escherichia coli*. *Nature* 406:64-7.

### Presentations

- Shigella: a phylogenetic study of the invasive Escherichia coli*, University of Maryland School of Medicine, Baltimore MD (July 21, 2009 – invited seminar)
- Mrowka, S.W., L. A. Parker, and **A. C. Bumbaugh**. 2007. Multi-Locus Variable Number Tandem Repeat (VNTR) subtyping of *Shigella sonnei* isolates, 83<sup>rd</sup> Annual Meeting of the Pennsylvania Academy of Science, Monroeville, PA.

- Slogenhop, D.L., J. B. Longnecker, J. A. Winsor and **A. C. Bumbaugh**. 2007. Molecular subtyping of *Erwinia tracheiphila*, a plant pathogen responsible for vascular wilt disease in cucurbits, 83<sup>rd</sup> Annual Meeting of the Pennsylvania Academy of Science, Monroeville, PA.
- Should Shigella be considered as a pathovar of Escherichia coli?: Molecular subtyping within the genus Shigella*, Microbiologists at Penn State, University Park, PA (November 7, 2007 – invited seminar)
- DNA-based subtyping methods: applications in the study of foodborne pathogens*, Department of Food Science, The Pennsylvania State University, University Park, PA (April 13, 2006 – invited seminar)
- Evolution of invasiveness in Escherichia coli and Shigella*, Foodborne & Diarrheal Diseases Branch, Centers for Disease Control and Prevention, Atlanta, GA (April 11, 2003 – invited seminar)
- Bumbaugh, A. C.**, R. F. Mangold, A. E. Plovanich-Jones, and T. S. Whittam. 2003. Genomic variation in *Shigella dysenteriae* type 1 using a new approach called paired end sequence mapping, 103<sup>rd</sup> General Meeting of the American Society for Microbiology, Washington, D.C.

### Grants, Awards, and Honors

- 2009 Undergraduate Research Fund, Shippensburg University, The detection of serine-protease autotransporter toxins (SPATe's) within the phylogenetic framework of the invasive *Escherichia coli*, co-authored with K. Simmons and D. Longenecker, \$500
- Undergraduate Research Fund, Shippensburg University, The investigation of repetitive element genome profiling to differentiate between isolates of the plant pathogen *Erwinia tracheiphila*, co-authored with K. Ryan and O. Belemou, \$1701
- Selected Participant, NSF Day, Indiana University of Pennsylvania, Indiana, PA (May 21)
- Changing the Teaching and Learning Paradigm Through the use of Technology Grant, Inquiry based learning utilizing image analysis in molecular biosciences, co-authored with S. Bergsten, L. Elliott, M. Lehman, and W. Patrie, \$20,937.60
- 2008 Invited Participant, ASM/BioQUEST Bioinformatics Institute, American Society for Microbiology Headquarters, Washington, DC (March 6-9, 2008)
- 2007 Research Development Grant, Penn State Altoona, O-antigen sequencing and analysis of a new serotype of pathogenic *Escherichia coli* associated with pigs, \$4350
- National Science Foundation Grant, Interrelationships Among Inbreeding Herbivory, and Disease Dynamics in a Wild Gourd, co-author with A. Stephenson and J. Winsor, submitted January 2007, resubmitted June 2007 (not funded)
- 2006 Research Development Grant, Penn State Altoona, *Molecular Subtyping and Epidemiology of the Plant Pathogen, Erwinia tracheiphila*, \$5000 (with J. Winsor)
- Undergraduate Research Award, *Molecular Subtyping and Epidemiology of the Plant Pathogen, Erwinia tracheiphila*, \$500 (to support D. Slogenhop)
- 2005 Dean's Development Fund, Penn State Altoona, *A preliminary population genetic study of Ferroplasma, an Archaean associated with acid mine drainage*, \$535
- Beckman Coulter's Genomic Educational/Research Matching Funds Program, \$49,500 (with L. Palmer, A. Parente, and J. Winsor)
- 2004 Dean's Development Fund, Penn State Altoona, *Multi-locus Variable Number Tandem Repeat Analysis to Identify Escherichia and Shigella Isolates*, \$2448

### Professional Societies

American Society for Microbiology, Allegheny Branch of the American Society for Microbiology, Pennsylvania Academy of Natural Sciences, Society for Molecular Evolution, American College and University Biology Educators, American Association for the Advancement of Science

## References

1. Bumbaugh, A. C., K. E. Hyma, D. W. Lacher, T. M. Large, L. M. Ouellette, C. L. Tarr, N. Strockbine, K. A. Talukder, D. A. Sack, and T. S. Whittam. Multilocus sequence divergence and gene acquisitions in the evolution of the enteroinvasive phenotype of *Escherichia coli* and *Shigella*, (In preparation)
2. DuPont, H. L., M. M. Levine, R. B. Hornick, and S. B. Formal. 1989. Inoculum size in shigellosis and implications for expected mode of transmission. *J Infect Dis* 159:1126-8.
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5. Faundez, G., G. Figueroa, M. Troncoso, and F. C. Cabello. 1988. Characterization of enteroinvasive *Escherichia coli* strains isolated from children with diarrhea in Chile. *J Clin Microbiol* 26:928-32.
6. Gordillo, M. E., G. R. Reeve, J. Pappas, J. J. Mathewson, H. L. DuPont, and B. E. Murray. 1992. Molecular characterization of strains of enteroinvasive *Escherichia coli* O143, including isolates from a large outbreak in Houston, Texas. *J Clin Microbiol* 30:889-93.
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10. Levine, O.S. and Levine M. M. 1991. Houseflies (*Musca domestica*) as mechanical vectors of shigellosis. *Rev Infect Dis* 13:688-96.
11. Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. *Emerg Infect Dis* 5:607-25.
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13. Pupo, G. M., D. K. Karaolis, R. Lan, and P. R. Reeves. 1997. Evolutionary relationships among pathogenic and nonpathogenic *Escherichia coli* strains inferred from multilocus enzyme electrophoresis and *mdh* sequence studies. *Infect Immun* 65:2685-92.
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